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Quantification of tamoxifen and three of its phase-I metabolites in human plasma by liquid chromatography/triple-quadrupole mass spectrometry

Lisette Binkhorst[a], Ron H.J. Mathijssen[b], Inge M. Ghobadi Moghaddam-Helmantel[b], Peter de Bruijn[b], Teun van Gelder[a], Erik A.C. Wiemer[b], Walter J. Loos[b,*]

[a] Department of Hospital Pharmacy, Erasmus MC, University Medical Center, Rotterdam, The Netherlands
[b] Department of Medical Oncology, Erasmus MC – Daniel den Hoed Cancer Center, University Medical Center, Rotterdam, The Netherlands

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ABSTRACT

In view of future pharmacokinetic studies, a highly sensitive ultra performance liquid chromatography/tandem mass spectrometry (UPLC–MS/MS) method has been developed for the simultaneous quantification of tamoxifen and three of its main phase I metabolites in human lithium heparinized plasma. The analytical method has been thoroughly validated in agreement with FDA recommendations. Plasma samples of 200 μl were purified by liquid–liquid extraction with 1 ml *n*-hexane/isopropanol, after deproteination through addition of 50 μl acetone and 50 μl deuterated internal standards in acetonitrile. Tamoxifen, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen were chromatographically separated on an Acquity UPLC® BEH C18 1.7 μm 2.1 mm × 100 mm column eluted at a flow-rate of 0.300 ml/min on a gradient of 0.2 mM ammonium formate and acetonitrile, both acidified with 0.1% formic acid. The overall run time of the method was 10 min, with elution times of 2.9, 3.0, 4.1 and 4.2 min for endoxifen, 4-hydroxy-tamoxifen, N-desmethyl-tamoxifen and tamoxifen, respectively. Tamoxifen and its metabolites were quantified by triple-quadrupole mass spectrometry in the positive ion electrospray ionization mode. The multiple reaction monitoring transitions were set at 372 > 72 (*m*/*z*) for tamoxifen, 358 > 58 (*m*/*z*) for N-desmethyl-tamoxifen, 388 > 72 (*m*/*z*) for 4-hydroxy-tamoxifen and 374 > 58 (*m*/*z*) for endoxifen. The analytical method was highly sensitive with the lower limit of quantification validated at 5.00 nM for tamoxifen and N-desmethyl-tamoxifen and 0.500 nM for 4-hydroxy-tamoxifen and endoxifen, which is equivalent to 1.86, 1.78, 0.194 and 0.187 ng/ml for tamoxifen, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen, respectively. The method was also precise and accurate, with within-run and between-run precisions within 12.0% and accuracy ranging from 89.5 to 105.3%. The method has been applied to samples from a clinical study and cross-validated with a validated LC–MS/MS method in serum.

1. Introduction

The selective estrogen receptor modulator tamoxifen remains an important drug in the treatment of estrogen receptor (ER) positive breast cancer. In the United States tamoxifen is also approved for the prevention of breast cancer in women at high-risk [1–4]. Tamoxifen reduces the risk of recurrence and the risk of mortality, however, not all women benefit from tamoxifen therapy, and treatment-related adverse reactions also vary greatly between patients. Inter-individual variability in metabolism of tamoxifen, which is influenced by both genetic and environmental factors, contributes to the differences in efficacy and toxicity of tamoxifen [1,2,5–7].

Tamoxifen is a prodrug and undergoes biotransformation into several metabolites, including N-desmethyl-tamoxifen, which is the most abundant metabolite, and its potent metabolites 4-hydroxy-tamoxifen and 4-hydroxy-N-desmethyl-tamoxifen (endoxifen). The cytochrome P450 enzymes CYP3A4 and CYP2D6 play a dominant role in the biotransformation of tamoxifen, with other CYP enzymes (CYP2B6, CYP2C9 and CYP2C19) playing a minor role [7–9]. The anti-estrogenic potency of 4-hydroxy-tamoxifen and endoxifen, regarding ER-binding and suppression of estrogen-dependent proliferation of breast cancer cells, is 30–100-fold higher compared with tamoxifen. As plasma concentrations of endoxifen are 5–10 times higher than of 4-hydroxy-tamoxifen, endoxifen is thought to be of most importance for the pharmacological activity of tamoxifen treatment [7,10,11].

Several studies have shown that genetic variation in CYP2D6 enzymes and the concomitant use of CYP2D6 inhibitors influence

* Corresponding author at: Erasmus University Medical Center – Daniel den Hoed Cancer Center, Department of Medical Oncology, Room AS-15, Groene Hilledijk 301, 3075 EA Rotterdam, The Netherlands. Tel.: +31 10 7041252; fax: +31 10 7041053.
E-mail address: w.loos@erasmusmc.nl (W.J. Loos).

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endoxifen plasma concentrations [7,12–14]. In addition, the activity of other CYP enzymes (CYP3A4/5, CYP2C9, and CYP2C19), which are also affected by genetic polymorphisms and concomitant medication, may also be responsible for the large inter-patient variability in endoxifen plasma concentrations. Therefore, monitoring endoxifen plasma concentrations rather than CYP2D6 genotype testing is suggested to be a better approach to personalize tamoxifen therapy.

To assess the effects of genetic polymorphisms in cytochrome P450 enzymes and influences of co-medication on the plasma concentrations of tamoxifen and its metabolites and for monitoring of endoxifen plasma concentrations, quantification of these compounds with a sensitive and validated analytical method is important. For this purpose, the development of bioanalytical methodologies for the quantification of tamoxifen and its metabolites in human serum, plasma, urine and tissue have been reported in various publications, reviewed by Teunissen et al. [15]. However, not all analytical assays included tamoxifen and its three main metabolites (N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen). In addition, not all assays have been thoroughly validated, which is important for its use in clinical pharmacokinetic studies and clinical practice [15].

Although a few LC–MS/MS assays have been adequately validated and included at least the three main phase I metabolites [16–18], the sensitivity of the methods may not be enough for the determination of low metabolite concentrations. One of these validated LC–MS/MS methods [16] was used for the quantification of tamoxifen and its metabolites in a recent study, in which dextromethorphan was used as a phenotyping probe to predict endoxifen exposure in patients using tamoxifen [19]. In several patients, serum levels of the tamoxifen metabolites 4-hydroxy-tamoxifen and endoxifen were below the lower limits of quantification of 1.13 and 2.69 ng/ml, respectively, and could not be reliable determined.

In view of future pharmacokinetic studies, we developed a highly sensitive and selective ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) assay for tamoxifen and its main phase I metabolites. The method requires only 200 μl plasma and involves a liquid–liquid extraction procedure for the purification of the plasma samples. The method is fully validated according to the Guidance for Industry, Bioanalytical Method Validation, as specified by the FDA, with lower limits of quantitation of 1.86, 1.78, 0.194 and 0.187 ng/ml for tamoxifen, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen, respectively.

2. Experimental

2.1. Chemicals

Pure Z (*cis*)-isomers of tamoxifen, N-desmethyl-tamoxifen and 4-hydroxy-tamoxifen, the stable labeled deuterated internal standards tamoxifen-d5, N-desmethyl-tamoxifen-d5, 4-hydroxy-tamoxifen-d5 and a racemic mixture of the Z- and E-isomers (1:1) of 4-hydroxy-N-desmethyl-tamoxifen-d5 were obtained from Toronto Research Chemicals (North York, ON, Canada). The pure Z (*cis*)-isomer of 4-hydroxy-N-desmethyl-tamoxifen (endoxifen) was kindly provided by Jina Pharmaceuticals Inc. (Libertyville, IL). All chemicals were of analytical grade or higher. Acetonitrile, methanol and water were from Biosolve BV (Valkenswaard, The Netherlands). Dimethylsulphoxide (DMSO), ammonium formate, glycine and *n*-hexane were purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands), sodium hydroxide and 2-propanol from Merck (Darmstadt, Germany) and formic acid from J.T. Baker (Deventer, The Netherlands). Blank human lithium heparinized plasma was obtained from Biological Specialty Corporation (Colmar, PA).

2.2. Preparation of stock solutions, calibration standards and quality control samples

Stock solutions containing 1.00 mM free base of tamoxifen, N-desmethyl-tamoxifen, 4-hydroxy-tamoxifen and endoxifen in DMSO were prepared individually. Following preparation, stock solutions were stored at $T < -70\,^{\circ}C$. Individual stock solutions of tamoxifen and its metabolites were used for the preparation of a working stock solution, containing 200 μM tamoxifen, 200 μM N-desmethyl-tamoxifen, 20 μM 4-hydroxy-tamoxifen and 20 μM endoxifen in DMSO. The working stock solution was divided into 150 μl aliquots, which were used for the construction of calibration curve standards during the validation. Separate stock solutions (i.e., independent weightings) of tamoxifen and its metabolites were used for the preparation of the pools of quality control samples. The variation between the stock solutions of tamoxifen and its metabolites used for the construction of the calibration standards and QC samples was in all cases <5%.

Deuterated internal standards were dissolved in DMSO separately, to obtain internal standard stock solutions at a concentration of 1 mg/ml free base, which subsequently were aliquotted and stored at $T < -70\,^{\circ}C$. Aliquots of 10 μl of the individual stock solutions were concurrently 10,000-fold diluted in acetonitrile, resulting in an internal standard working solution containing 100 ng/ml tamoxifen-d5, N-desmethyl-tamoxifen-d5, 4-hydroxy-tamoxifen-d5 and 4-hydroxy-N-desmethyl-tamoxifen-d5, which was stored at $T < 8\,^{\circ}C$ for a maximum of 3 months.

Calibration curve standards were freshly prepared (in duplicate) for each run, by addition of 10 μl aliquots of appropriate dilutions of the working stock solution in acetonitrile/DMSO (1:1, v/v) to 190 μl aliquots of human lithium heparinized plasma (excepted of calibration standard 7, which was prepared by addition of 45 μl diluted working stock solution to 955 μl plasma) at the following concentrations: 5.00, 10.0, 50.0, 100, 250, 500, 900, and 1000 nM for tamoxifen and N-desmethyl-tamoxifen and 0.500, 1.00, 5.00, 10.0, 25.0, 50.0, 90.0, and 100 nM for 4-hydroxy-tamoxifen and endoxifen.

A total of five pools of quality control (QC) samples were prepared by spiking appropriate dilutions of stock solutions of tamoxifen and its metabolites to human lithium heparinized plasma at concentrations of 5.00 nM (LLQ), 15.0 nM (QC-Low), 400 nM (QC-Middle), 800 nM (QC-High) and 16,000 nM (QC-Diluted) for tamoxifen and N-desmethyl-tamoxifen and at 0.500 nM (LLQ), 1.50 nM (QC-Low), 40.0 nM (QC-Middle), 80.0 nM (QC-High) and 1,600 nM (QC-Diluted) for 4-hydroxy-tamoxifen and endoxifen. QC-Diluted was processed after a 20-fold dilution in blank human lithium heparinized plasma. Pools of QC samples were aliquotted and stored at $T < -70\,^{\circ}C$ until analysis.

2.3. Plasma sample preparation

Aliquots of 50 μl of internal standard working solution and 50 μl of acetone were added to 200 μl of plasma samples in 1.5 ml microcentrifuge tubes and vigorously vortexed for 5 min. The samples were then centrifuged at 18,000 × *g* at ambient temperature for 10 min. Subsequently, the supernatant was transferred into 2 ml microcentrifuge tubes and 100 μl aliquots of glycine buffer (pH 11.5) and 1 ml aliquots of n-hexane/2-propanol (95:5, v/v) were added. Hereafter, the samples were again vortexed and centrifuged under the previously mentioned conditions. Aliquots of 800 μl of the organic phase were transferred into 4.5 ml glass tubes and evaporated to dryness under nitrogen at $T = 60\,^{\circ}C$. The residues were reconstituted in 100 μl aliquots of acetonitrile/water/formic acid